Evaluation of Real-Time PCR for Detection of and Discrimination between *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella holmesii* for Clinical Diagnosis

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PCR is increasingly being used as a diagnostic test for the detection of *Bordetella pertussis* and *Bordetella parapertussis* DNA, as it has improved sensitivity and specificity in comparison to conventional techniques. The assay described here uses the two insertion sequences IS*481* and IS*1001* for *B. pertussis* and *B. parapertussis*, respectively, with detection by molecular beacons. The real-time PCR for IS*481* detects both *B. pertussis* and *Bordetella holmesii*, and the real-time PCR for IS*1001* detects both *B. parapertussis* and *B. holmesii*. By performing both assays discrimination between *B. pertussis* and *B. parapertussis* can be obtained. The sensitivity was 1 to 10 CFU/ml for *B. pertussis*, 10 CFU/ml for *B. parapertussis*, and 10 CFU/ml for *B. holmesii* in both assays. The clinical sensitivity of the *B. pertussis* assay was not affected by duplexing with an internal control PCR. Real-time PCR, conventional PCR, and culture were performed on 57 clinical samples. Eight of the 57 (14%) were found positive by culture, 19 of 57 (33%) were found positive by conventional PCR, and 22 of 57 (39%) were found positive by real-time PCR. One sample was inhibitory. When the *B. pertussis* assay was compared with a clinical standard for *B. pertussis* infection, sensitivity was 38, 83, and 100% and specificity was 100, 97, and 97% for culture, conventional PCR, and real-time PCR, respectively. The real-time PCR designed for *B. pertussis* and *B. parapertussis* provides sensitive and specific diagnosis of *B. pertussis* and *B. parapertussis* infections and is therefore suitable for implementation in the diagnostic laboratory.

Bordetella pertussis is the causative agent of whooping cough (pertussis), an infectious disease that occurs worldwide with a high prevalence among young unvaccinated infants (1, 27) and recently has reemerged in highly vaccinated populations (10). Pertussis-like symptoms are also caused by related species of bacteria, including *Bordetella parapertussis* and *Bordetella holmesii* (16), although *B. parapertussis* infections are usually less severe (8).

Laboratory diagnosis is traditionally based on culture, which is regarded as the "gold standard" and is highly specific but whose sensitivity in comparison to PCR or serology varies between 7 and 36% (13, 29, 31). Furthermore, culturing depends on specimen quality, rapid transportation, and laboratory expertise. This is an important factor in all diagnostic assays requiring propagation of the pathogen, but in the case of *B. pertussis* these requirements may be more acute. Adequate serological detection of *B. pertussis* generally requires paired samples, although immunoglobulin A or immunoglobulin G measurements in a single serum can also be used (2). So far no serologic assay has been approved for diagnostic use in the United States because diagnostic criteria have not been accepted and no method has been validated between laboratories (22).

PCR is increasingly being used as a diagnostic test for the

detection of *B. pertussis* and *B. parapertussis* DNA (4, 7, 18, 26, 30). In a comparison of different PCR assays used in pertussis vaccine studies, all assays provided an increased sensitivity for detection of pertussis cases by at least 70% in comparison to culture (23). Various PCR protocols have been developed that target different regions of the genome: insertion sequences IS*481* and IS*1001* (4, 6, 13, 15, 26, 30), the pertussis toxin promoter region (6, 15, 20, 26), the porin gene (5), and the adenylate cyclase gene (3). The achievements of the PCR assays targeting these different genes have not been widely compared. The insertion sequences have been found to be more sensitive than the pertussis toxin promoter (26), but other workers have shown comparable sensitivities for the insertion sequences and the porin gene (5) and the insertion sequences and the pertussis toxin promoter (25). The copy number of the insertion sequence IS*481* in *B. pertussis* is approximately 100, the copy number of the insertion sequence IS*1001* in *B. parapertussis* is 20 to 21 (32), and for *B. holmesii* the copy number is unknown. Recently, diagnostic applications of PCR formats have been significantly improved by implementing fluorescent, real-time detection of the amplification products. By adding fluorescent probes to the amplification reaction PCR products can be detected as they are generated. For qualitative PCR applications real-time PCR offers advantages, as post-PCR processing is redundant. This reduces the time to results and in addition the risk of contamination. Some real-time PCR assays have been described for *B. pertussis* (11, 21, 26). For appropriate application of these PCR assays consensus recommendations have been formulated (17), and so far only one conven-

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TABLE 1. Bacterial species and strains

Species	Strain or source	Origin[a]
Bordetella pertussis	ATCC 9797	ATCC
Bordetella parapertussis	ATCC 15311	ATCC
Bordetella bronchiseptica	ATCC 19395	ATCC
Bordetella holmesii	ATCC 51541	ATCC
Bordetella avium	LMG 1851	UZA
Bordetella hinzii	LMG 1872	UZA
Bordetella trematum	LMG 5894	UZA
Mycoplasma pneumoniae type 1	ATCC 29085 (PI 1428)	ATCC
Mycoplasma pneumoniae type 2	ATCC 15492 (MAC)	ATCC
Ureaplasma urealyticum	Clinical isolate	UZA
Legionella pneumophila	ATCC 33152	ATCC
Chlamydia pneumoniae	TW-183	
Moraxella catarrhalis	ATCC 25238	ATCC
Haemophilus influenzae	ATCC 43065	ATCC
Streptococcus pneumoniae	ATCC 49150	ATCC
Streptococcus pyogenes	ATCC 12344	ATCC
Enterococcus faecalis	ATCC 12984	ATCC
Staphylococcus aureus	ATCC 12600	ATCC
Klebsiella pneumoniae	ATCC 13883	ATCC
Escherichia coli	ATCC 11775	ATCC
Neisseria meningitidis	ATCC 13090	ATCC
Pseudomonas aeruginosa	ATCC 10145	ATCC
Enterobacter aerogenes	ATCC 13048	ATCC

[a] ATCC, American Type Culture Collection; UZA, Universitair Ziekenhuis Antwerpen.

tional PCR assay has been described that fulfills all these recommendations (5).

Here, we describe the development and application of a real-time PCR format using molecular beacons to detect and distinguish between *B. pertussis* and *B. parapertussis* in a test format including an internal control which complies with the consensus recommendations.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used to test the specificity of the real-time PCR are presented in Table 1. All bacteria used for specificity testing were cultured by standard methods. In addition a further 10 culture-positive isolates of *B. pertussis*, 5 culture-positive isolates of *B. parapertussis*, and two *B. holmesii* PCR-positive samples were selected from our repository and included in the analyses. The *B. holmesii* clinical samples were detected using the PCR described by van der Zee et al. (30)

To evaluate the sensitivity of the PCR test, *B. pertussis*, *B. parapertussis*, and *B. holmesii* were suspended in sterile physiological saline at a concentration equivalent to 10^8 cells per ml, based on McFarland turbidimetric standards. Serial dilutions were made from the suspension, and aliquots were extracted and tested in the PCR to determine sensitivity.

Collection and processing of samples. From April 2001 to December 2001 specimens were obtained from 57 patients who were investigated for *B. pertussis* infection. Patients presented with cough or wheezing. Nasal swabs (12 samples), throat swabs (37 samples), sputum (3 samples), and nasopharyngeal aspirates (NPAs) (5 samples) were received. Swabs from patients were obtained either in charcoal-based Stuart's transport medium with a Dacron swab, in transport medium, or as dry swabs. The swab used for the transport medium and the dry swab was a cotton-tipped swab, and these samples were used for PCR only. The buffered transport medium contained 0.0185% CaCl$_2$, 0.8% NaCl, 0.04% KCl, 0.02% MgSO$_4$, 0.0075% Na$_2$HPO$_4$, 0.1% glucose, 0.5% albumin, 0.001% phenol red, 0.1% yeast extract, 0.5% gelatin, and 0.0025% vancomycin. NPAs and sputum samples were mixed well by vortexing for 30 s, and aliquots were frozen at $-70°C$. Swabs were placed in 0.5 ml of 0.9% NaCl prepared in RNase- and DNase-free water, swirled vigorously, and wrung out, and the swab was removed from the specimen before freezing. All specimens were stored at $-70°C$ prior to nucleic acid isolation. All 57 patients included had samples suitable for culture and PCR. Retrospectively, clinical data for these 57 patients were collected. For

some cases serological data (2) were obtained by an in-house assay using pertussis toxin or a sonicated whole bacterium as antigen available from the National Institute of Public Health (Bilthoven, The Netherlands). As a negative-control group throat swabs were collected from 20 children with mild respiratory symptoms but with no presence of cough or fever.

Bacterial culture. All specimens suitable for culture were inoculated within 24 h of collection onto Regan-Lowe medium without antibiotics and Regan-Lowe medium with cephalexin. Plates were incubated at 35°C in a moist Perspex chamber containing air with adequate humidity for up to 7 days. Plates were examined daily for suspect colonies which were identified phenotypically. Suspect colonies for *B. pertussis* and *B. parapertussis* or *B. holmesii* were further investigated with agglutinating serum (Murex, Dartford, England).

DNA extraction. Nucleic acids were extracted from bacterial suspensions and clinical specimens with the QiaAmp DNA blood minikit (Qiagen, Hilden, Germany). All samples were extracted according to the manufacturer's instructions with 200 μl of samples in each extraction. Negative no-template controls were included in each run and after every 20 samples. In the control sterile distilled water was added instead of specimen.

Primers and probe for conventional PCR. PCR amplification for *B. pertussis* was performed using primers which amplified a 181-bp region of IS481 (accession no. L26973). PCR amplification for *B. parapertussis* was performed using primers that amplified a 153-bp region of the IS1001 (accession no. X66858). Primers and probe sequences are shown in Table 2.

Conventional PCR. Briefly, the PCR was performed in 50 μl of reaction mixture consisting of 25 μl of *Taq* PCR Master Mix (Qiagen), 1.5 mM MgCl$_2$, 0.2 μM (each) primer, and 20 μl of extracted DNA. The PCR thermal profile consisted of an initial incubation of 3 min at 94°C; followed by 40 cycles of 15 s at 94°C, 20 s at 60°C, and 30 s at 72°C; and finally a 10-min hold at 72°C. The *B. pertussis* product was captured in an avidin-coated enzyme-linked immunosorbent assay plate and subsequently detected by enzymatic reaction with a probe specific to the IS481 product (34). The probe was labeled with digoxigenin. Inhibition was determined by spiking samples with a *B. pertussis* DNA. The *B. parapertussis* product was detected by agarose gel electrophoresis. Inhibition was determined by spiking duplicate PCRs with *B. pertussis* or *B. parapertussis* DNA.

Sequencing of IS1001 for *B. parapertussis* and *B. holmesii*. A PCR product was obtained for both *B. parapertussis* and *B. holmesii* by using the primers and PCR as previously described by van der Zee et al. (30). These primers detect both *B. parapertussis* and *B. holmesii*. Nucleotide sequence analysis was set up in 20-μl reaction volumes with a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Warrington, United Kingdom) according to the manufacture's specifications. Briefly, the reaction mixture contained 3.0 pmol of either PCR primer and purified template DNA (1 μl is approximately equal to 200 to 500 ng of DNA). After cycle sequencing the products were purified using an isopropanol precipitation and subsequently analyzed on the ABI 310 automated DNA sequencer (Applied Biosystems). Analysis of the sequences was performed using Vector NTI (Informax).

Primers and probes for real-time PCR. Primer and molecular beacon sequences were selected from the sequence of *B. pertussis* IS481 (accession no. L26973) by using criteria required for design of molecular beacon assays. This region is 99% homologous to the same region in *B. holmesii* (accession no. AF349431). Primer and molecular beacon sequences were selected from sequence *B. parapertussis* IS1001 (accession no. X66858) and from the sequencing data for the IS1001 region of both *B. holmesii* and *B. parapertussis*. The real-time PCR was designed so that both *B. holmesii* and *B. parapertussis* would be detected by the assay. All PCR primers were designed using the Primer 3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) to ensure minimal self-complementary and no secondary structures.

The molecular beacon was designed using the Mfold Zuker program (http://www.bioinfo.rpi.edu/applications/mfold/old/dna/). Additional criteria for a good molecular beacon included a melting temperature of 8°C over the melting temperature of the primers and the amplicon being relatively short, <150 bp. The stem sequence was selected to have a melting temperature compatible with the molecular beacon. The beacon formed a stable structure at 50 to 55°C, the proposed annealing temperature, with no secondary structures. A BLAST search was performed to check the specificity of the DNA sequences of the primers and probes. For *B. pertussis* the fluorescent reporter on the 5′ end of the probe was 6-carboxyfluorescein and the quencher on the 3′ end was Dabcyl. For *B. parapertussis* the fluorescent reporter on the 5′ end of the probe was Texas Red and the quencher on the 3′ end was Black Hole Quencher 2. The molecular beacon and primers were prepared by Biolegio (Malden, The Netherlands). Selected primers and probe are shown in Table 2.

Real-time PCR. Real-time PCR for *B. pertussis* was performed in 50 μl of reaction mixture consisting of 25 μl of Hotstar *Taq* Master Mix (Qiagen), 3.5 mM

TABLE 2. Primers and probes for PCR

Assay format and target	Primer or probe sequence[a]	Primer or probe	Amplicon size (bp)
PCR			
IS*481*	GATTCAATAGGTTGTATGCATGGTT	Forward primer	181
	TTCAGGCACACAAACTTGATGGGCG	Reverse primer	
	CGTCGACTCGAAATGGTCCAGC	Probe	
IS*1001*	CGCTGGCTGCTGCTGCGCAA	Forward primer	171
	GTGGTTCCACGCTTGTCTTG	Reverse primer	
RT-PCR[b]			
IS*481*	TCAATAGGTTGTATGCATGG	Forward primer	154
	GATCAATTGCTGGACCATT	Reverse primer	
	FAM-<u>GCAGGC</u>GGCCGGATGAACACCCATAA<u>GCCTGC</u>-Dabcyl	Molecular beacon	
IS*1001*	CCATGTCGTGGCCAAGTA	Forward primer	186
	TGGTTGGCTTGCAGCAA	Reverse primer	
	Texas red-<u>GCAGGC</u>GCTGGCTRCTGCTGCGCAA<u>GCCTGC</u>-BHQ2	Molecular beacon	
PhHV gB	ATGCATTTAAAACCCTCAAA	Forward primer	140
	GCATCAACTTCTTCGACAAT	Reverse primer	
	Cy-5-<u>GTCGCC</u>CCTGGTTTTTATCGTACGGGAACA<u>GGCGAC</u>-BHQ2	Molecular beacon	

[a] Underlined nucleotides indicate the stem structure of the molecular beacon.
[b] RT-PCR, real-time PCR.

MgCl$_2$, 0.1 μM (each) primer, 0.34 μM molecular beacon, and 10 μl of template. For *B. parapertussis* the real-time PCR was performed in 50 μl of reaction mixture consisting of Hotstar *Taq* Master Mix, 5.5 mM MgCl$_2$, 0.2 μM (each) primer, 0.45 μM molecular beacon, and 10 μl of template The PCR thermal profile consisted of an initial incubation of 15 min at 95°C followed by 50 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. Amplification, detection, and data analysis were performed with an iCycler IQ Real-Time Detection system (Bio-Rad, Veenendaal, The Netherlands).

Internal control real-time PCR. The quality of nucleic acid extraction as well as inhibition of the real-time PCR was monitored by amplification of a phocine herpesvirus (PhHV) spike. This method has been described by Niesters using Taqman probes (19). For implementation in the *B. pertussis* real-time PCR the assay was redesigned using primer and molecular beacon as a probe, with the molecular beacon labeled with Cy-5 (28). The PhHV-optimized primer and probe concentrations were used in the *B. pertussis* real-time PCR. Each sample was spiked with a dilution of viral stock of PhHV which was coextracted with the sample, and the assay was performed as a duplex PCR.

Inter- and intra-assay variability. DNA was extracted from *B. pertussis* ATCC 9797 and stored in AE buffer. The DNA was diluted to a concentration equivalent to 100 CFU/ml and stored in small aliquots at −20°C. In order to determine inter- and intra-assay variation an aliquot was thawed and run in quintuplicate in five consecutive runs of the multiplex real-time PCR assay.

Analysis of results. All clinical data and laboratory results were coded and entered into a database. A pertussis case was defined as cough lasting at least 2 weeks, paroxysms of coughing or vomiting, and one or more of the following symptoms or characteristics: apnea or cyanosis, subconjunctival bleeding, lymphocytosis, or a recent contact (up to 3 weeks) with a whooping cough patient. In addition to these clinical criteria whooping cough was also considered in the case of (i) positive culture of *B. pertussis* or (ii) serological confirmation according to the criteria of the National Institute of Public Health (2).

The clinical sensitivities and specificities and predictive values of the results were determined in two frequency tables with the clinical criteria for pertussis as the gold standard.

RESULTS

Real-time PCR for *B. pertussis*. The real-time PCR for *B. pertussis* was optimized first as a monoplex assay with a 10-fold dilution series of both *B. pertussis* DNA and *B. holmesii* DNA. PCR efficiency with the iCycler software was 98 and 95% for *B. pertussis* and *B. holmesii*, respectively. The monoplex real-time PCR was thereafter compared to duplex PCR with the PhHV internal control. The threshold cycle (Ct) values obtained in the monoplex assay and the duplex assay showed a mean difference of values of 0.3. In addition the clinical sensitivity of the assay was not affected by duplexing.

Sequence analysis of IS*1001* for *B. holmesii* and *B. parapertussis*. A 440-bp product amplified from the IS*1001* fragment of both *B. holmesii* and *B. parapertussis* was sequenced. The resulting sequences were aligned. Based on the consensus regions a real-time PCR for both targets was designed. There was approximately 87% homology between the two *Bordetella* spp. The sequence data were compared to sequence data from the whole IS*1001* for both *B. parapertussis* and the equivalent region for *B. holmesii*, and there was good correlation between the sequencing results. From the sequence data it appears that this sequence is related to an insertion sequence for *B. holmesii*.

Real-time PCR for *B. parapertussis*. The real-time PCR for *B. parapertussis* was optimized as a monoplex assay in the same way as the *B. pertussis* assay. PCR efficiency with the iCycler software was 102 and 105% for *B. parapertussis* and *B. holmesii*, respectively. Both assays had the same PCR protocol, so they could be performed in a single plate.

Specificity. Primers and probes of the *B. pertussis* real-time PCR assay successfully amplified DNA from both *B. pertussis* and *B. holmesii* and none of the other *Bordetella* species or other respiratory bacteria listed in Table 1. Additionally 10 positive isolates for *B. pertussis* and two clinical samples positive for *B. holmesii* were detected by the real-time PCR assay. The primers and probes from the *B. parapertussis* real-time PCR assay successfully amplified DNA from *B. parapertussis* and *B. holmesii* and none of the other *Bordetella* species or other respiratory bacteria listed in Table 1. Additionally, the five *B. parapertussis*-positive isolates and two clinical samples positive for *B. holmesii* were detected by the real-time PCR assay. Application of the results to diagnose infection by *B. pertussis* and *B. parapertussis* is summarized in Table 3.

TABLE 3. Use of assays to determine diagnosis

Diagnosis	Result by RT-PCR[a]		
	B. pertussis	B. parapertussis	Internal control
B. pertussis	Pos	Neg	Pos
B. holmesii or coinfection[b]	Pos	Pos	Pos
B. parapertussis	Neg	Pos	Pos
Valid result, negative for B. pertussis, B. parapertussis, and B. holmesii	Neg	Neg	Pos
Inhibition or invalid extraction—test needs to be repeated	Neg	Neg	Neg

[a] RT-PCR, real-time PCR; Pos, positive; Neg, negative.
[b] Differentiation by Sequencing of real-time PCR product.

Sensitivity. DNA was extracted from dilution series of the B. pertussis suspensions and subjected to PCR amplification. A sample was interpreted as positive if the relative fluorescence crossed the threshold as determined by the iCycler detection system software. The sensitivity of the real-time PCR assays was shown to be 1 to 10 CFU/ml, which was similar to that of the conventional PCR. B. holmesii could be detected with a sensitivity of 10 CFU/ml. The sensitivity of the B. parapertussis real-time PCR assay, applied to a dilution series of B. parapertussis and B. holmesii suspension, was determined to be 10 CFU/ml for both targets.

Evaluation of PCR inhibition. For application of PCR to clinical specimens in a diagnostic laboratory sufficient controls should be included. Besides specific positive and negative PCR controls, DNA isolation and inhibition should be monitored as well. In this real-time assay the PhHV was used as an internal control.

A dilution series of PhHV from 10^8 to 10^1 PFU of PhHV/ml was coamplified with the B. pertussis targets, which did not significantly affect the efficiency of the reaction. In addition, a spike of 10^3 PFU of PhHV/ml in the sample prior to extraction resulted in a positive result at a Ct value of 33 to 34, and this was not significantly affected by amplification of a dilution series of B. pertussis and resulted in the same clinical sensitivity. Thus, spiking and coamplification of PhHV and the Bordetella targets resulted in similar Ct values in comparison to amplification without spikes (Table 4).

Reproducibility of B. pertussis real-time PCR assay. Samples containing 100 CFU/ml were assayed in quintuplicate in the multiplex assay to determine the interassay and intra-assay variability. As determined from the Ct values obtained from five consecutive runs the interrun variation was 0.7 for this standard sample. The mean of the intra-assay variability was 0.36 (range, 0.2 to 0.6).

TABLE 4. Ct values for B. pertussis CFU-per-milliliter dilution series with and without the PhHV spike

Dilution (CFU/ml)	Ct value	
	No spike	With spike
10,000	27.5	28
1,000	32.1	33
100	35.9	36.2
10	39	40.2
1	45.5	46.7

TABLE 5. Results of culture, conventional PCR, and real-time PCR from 57 patient samples with clinically suspected pertussis infection[a]

Method	No. (%) positive			No. with result in clinical pertussis test	
	B. pertussis	B. parapertussis	B. holmesii	Pos	Neg
Culture	8 (14)	0	0	8	13
Conv. PCR	18 (33)	0	NT	17	3
RT-PCR	22 (39)	0	0	21	0
Total	57	57	57	21	

[a] Abbreviations: Pos, positive; Neg, negative; Conv. PCR, conventional PCR; RT-PCR, real-time PCR; NT, not tested.

Clinical evaluation of real-time PCRs. During the 8-month period described, 57 patients were seen and clinically suspected of Bordetella infection. Twenty-one of these 57 fulfilled the clinical definition for pertussis infection. Real-time PCR, conventional PCR, and culture were performed on clinical samples of these patients. Eight of the 57 (14%) were found positive by culture, 19 of 57 were found positive by conventional PCR (33%), and 22 of 57 (39%) were found positive by real-time PCR (Table 5). One sample was inhibitory, as the PhHV spike was not amplified. This sample was also inhibitory by conventional PCR and was culture negative (serology was not available). When the B. pertussis assays were compared with the clinical standard for B. pertussis infection, the sensitivity was 38, 85, and 100%; the specificity was 100, 97, and 97%; the positive predictive value was 100, 93, and 95%; and the negative predictive value was 73, 92, and 100% for culture, conventional PCR, and real-time PCR, respectively. As shown, there were three samples positive by real-time PCR only, and all had clinical criteria for disease (Fig. 1). One of the 11 samples positive by both B. pertussis PCR assays did not conform to clinical disease criteria (Fig. 1). Individual sensitivities
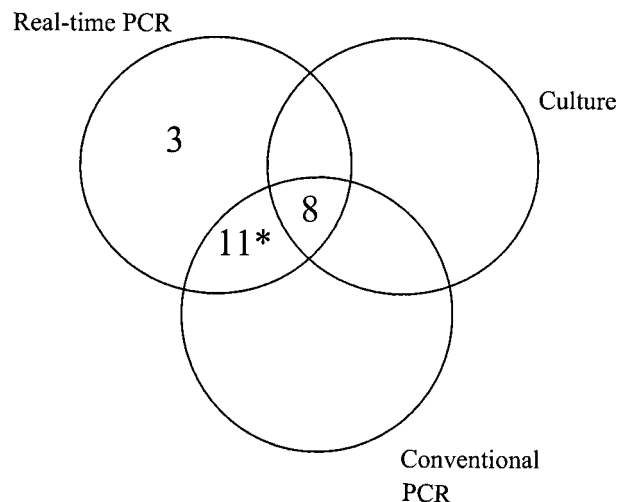

FIG. 1. Venn diagram showing assays demonstrating B. pertussis positivity. One sample positive by both PCR assays (*) did not conform to the clinical definition of pertussis.

and specificities for different samples were assessed for culture and real-time PCR. The sensitivity for culture in throat swab, nasal swab, sputum, and NPAs was 18, 16, 100, and 100%, respectively, and the specificity for all samples was 100%. The sensitivity for real-time PCR was 100% for all samples, and the specificity was 100% for all samples except throat swabs, which had a specificity of 97%. The mean Ct value for the patients with a positive culture was 28.8 (range, 17.1 to 34.7), and for patients with no positive culture the mean was 40.3 (range, 33.3 to 46). None of the 57 patients were positive for either *B. parapertussis* or *B. holmesii* by any of the methods.

DISCUSSION

The real-time PCR assay described provides an effective way to detect and discriminate between *B. pertussis* and *B paraper-tussis* infection. PCR has repeatedly been shown to be more sensitive than culture and direct fluorescent-antibody assay for detection of *B. pertussis* (7, 13, 23, 29, 30, 33). However, anal-ysis of discrepant results and assessment of clinical perfor-mance of PCR have been carried out less frequently (13, 33). The real-time PCR assays have excellent analytical sensitivity as shown previously for other PCR methods (11, 13, 26). PCR is much more sensitive than culture because culture requires viable microorganisms. *B. pertussis* is a particularly labile or-ganism, and most diagnostic laboratories are unable to culture the patient's material directly from the patient. In this study culture sensitivity was 38%, but others have reported rates of 15.2% (13) and 73.4% (33). The difference in sensitivity rates can be explained by differences in transportation to the labo-ratory, differences in when the sample is inoculated onto agar, and also the age of the patients. For example, Wadowsky et al., who achieved a culture sensitivity of 73%, immediately inocu-lated the samples onto agar after collection and tested a mainly pediatric population (33). A comparison of sample types showed that NPAs and sputum were better for culture, but the numbers were small, so conclusions are limited. In this study it appears that the real-time PCR has greater sensitivity than conventional PCR, as three clinical samples were positive in the real-time PCR only. Inclusion of the molecular beacon in the real-time assay may create a more stable template for the PCR, hence providing improved efficiency and thus sensitivity. In addition, owing to the software analysis with real-time PCR, which calculates efficiency, a more optimal reaction is achieved with the real-time assay than with conventional PCR assays. This also improves the sensitivity of the real-time PCR. Sam-ples with lower Ct values in the real-time PCR assay were more likely to be culture positive and thus have a higher concentra-tion of bacteria. The mean Ct in culture-positive samples was 28.8 compared with a mean Ct of 40 in culture-negative sam-ples, providing further evidence that the PCR assay had in-creased sensitivity to culture.

In this study the real-time PCR assays were performed in two tubes, although a multiplex format detecting all three *Bordetella* targets and the internal control in a single tube was possible with similar sensitivities for *B. pertussis* and *B. para-pertussis* (results not shown). The sensitivity of *B. holmesii* was 3 logs less in the multiplex format than in the two-well format. As the *B. holmesii* DNA had to be detected by both molecular

beacons, this may have affected the efficiency of the multiplex format.

A PhHV spike was added to the clinical samples prior to DNA isolation and coamplified in the *B. pertussis* real-time PCR assay. In this way, control of the DNA isolation proce-dure as well as a check for inhibition was achieved. The level of inhibition was low, as only 1 in 57 samples (2%) was inhibited. Hence, the use of the extraction with guanidine thiocyanate to lyse cells, followed by washing and precipitation of nucleic acids, is very effective at removing PCR inhibition, although when sputum samples are used more inhibition can be ex-pected (9). In this study, swabs were the main samples col-lected, and other studies using only dry swabs have also found very low rates of inhibition (13).

The positive predictive value of culture and *B. pertussis* real-time PCR was 100 and 95%, respectively, using the clinical criteria as the gold standard. This value is very similar to that of Loeffelholz et al. (13). The one PCR-positive patient not meeting the criteria did have a paroxysmal cough. However, serology was not performed and *B. pertussis* data for pertussis in the local environment were not available, so it may be that this patient actually had *B. pertussis* infection but that the information to assess by clinical criteria was unavailable.

When PCR is used in the diagnostic laboratory, concerns remain regarding false-positive results due to contamination or cross-reactivity. The use of real-time PCR helps to minimize the risk of false positives, as there is no need to handle ampli-fied material. In this study all samples were also tested by a second conventional PCR, which, although targeting the same repeat region, had different primers and probe, and the se-quences did not overlap. Nineteen of the 22 positives were confirmed by this method. The other three all met clinical criteria for disease. Confirmation of positives, especially in an outbreak situation, by a second PCR is recommended (12). In addition, consensus recommendations require the use of an alternative PCR to confirm questionable results (17). In the present study all samples were run with negative controls and all sample processing and testing were performed according to the regulations in the laboratory, which prevent PCR contam-ination. None of the negative controls were positive, and also each sample with a positive signal was repeatedly positive.

The clinical significance of detecting *B. holmesii* is not yet fully understood, but the bacterium has been isolated from the respiratory tract (16, 35) and been reported to cause disease (24). However, because of the design of the PCR assays de-scribed it is possible to provide a presumptive diagnosis for *B. holmesii*, as both assays detect *B. holmesii*. However, a mixed infection of *B. pertussis* and *B. parapertussis* is also possible but rare, and to date only one has been reported (11). Essentially any PCR assay targeting the IS*481* region will detect *B. pertus-sis* and *B. holmesii* (22), so the use of the *B. parapertussis* PCR, which detects both *B. holmesii* and *B. parapertussis*, enables *B. holmesii* infection or a dual infection of *B. pertussis* and *B. parapertussis* to be diagnosed. In the rare event that this occurs, the PCR product from the *B. parapertussis* PCR can be sequenced and *B. holmesii* infection can be distinguished from a dual infection. Of other *Bordetella* species such as *B. bron-chiseptica*, *B. avium*, *B. trematum*, and *B. hinzii*, it is only *B. bronchiseptica* that has been reported to cause respiratory infection in immunocompromised hosts (14). So the possibility

of this pathogen in immunocompromised patients should be considered separately.

Molecular methods have been shown to be highly sensitive and specific for *B. pertussis*, but one standardized method has yet to be adopted, although consensus recommendations have been published (17). Only the assay published by Farrell et al. complies with these recommendations, except that it does not distinguish between *B. parapertussis* and *B. bronchiseptica* (5). The assay described here complies with all the recommendations, except that the confirmatory PCR, although it uses different primers, targets the same IS*481* sequence.

The real-time PCR described here for *B. pertussis* and *B. parapertussis* provides sensitive and specific diagnosis of *B. pertussis* and *B. parapertussis* infections. The protocol used was able to complete analysis of 30 samples including controls in 4 to 5 h, including extraction and assay time, and therefore is suitable for implementation in the diagnostic laboratory.

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